UNIVERZITA KARLOVA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra farmakologie a toxikolgie

DIPLOMOVÁ PRÁCE

Stanovení role lysofosfatidylinositolu, GPR55 agonisty, u mechanicky způsobené bolesti

Vypracováno na Institutu farmakologie

Oddělení molekulární farmakologie

Ruprecht-Karls-University

Heidelberg

Školitelé:

Prof. Dr. Rohini Kuner

Doc. PharmDr. Petr Pávek, Ph.D.

Hradec Králové, 2.5. 2011

Dagmar Škoricová

CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Pharmacology and Toxicology

DIPLOMA THESIS

Dissecting the role of GPR55 agonist lysophosphatidylinositol in mechanically evoked pain

Performed at

Institute of Pharmacology

Department of Molecular Pharmacology

Ruprecht-Karls-University

Heidelberg

Supervisors:

Prof. Dr. Rohini Kuner

Doc. PharmDr. Petr Pávek, Ph.D.

Hradec Králové, 2.5. 2011 Dagmar Škoricová

PROHLÁŠENÍ

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a jsou v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

DECLARATION

I declare that this work is my original author work, which I had developed by myself. All literature and other sources, which I had used, are given in the list of used literature and they are quoted in text regularly. The work has not been used for achievement of identical or any other academic degree.

Hradec Králové, 2.5. 2011 .....

Dagmar Škoricová

ACKNOWLEDGEMENTS

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Dagmar Škoricová

ABSTRAKT

Univerzita Karlova v Praze

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Kandidát: **Dagmar Škoricová**

Školitelé: **Prof. Dr. Rohini Kuner**

Doc. PharmDr. Petr Pávek, Ph.D.

Název diplomové práce: **Stanovení role lysofosfatidylinositolu, GPR55 agonisty, u mechanicky způsobené bolesti.**

Nedávné studie naznačují, že sirotčí receptor GPR55 (receptor spojený s G-proteinem 55), který je aktivován několika různými kanabinoidními ligandy a také lysofosfolipidem L-α-lysofosfatidylinositolem (LPI), může být nový kanabinoidní receptor.

GPR55 je podle studií exprimován na senzorických neuronech a u GPR55 deficientních myší byla prokázána snížená mechanická hypersenzitivita v modelech zánětlivé a neuropatické bolesti. To společně naznačuje možnou pronocicepční roli receptoru GPR55. Bylo také zjištěno, že LPI je vylučován ve velkém množství z nádorových tkání. To nás vedlo k domněnce, že LPI uvolněné z rakovinových buněk může vést k sensitizaci nociceptorů a tím k rakovinou-indukované bolesti.

Cílem této práce bylo zjistit úlohu LPI v onkologické, mechanicky indukované bolesti a její možný mechanismus. Zjistili jsme, že neurony ganglií zadních kořenů míšních jsou *in vitro* přímo aktivované LPI. Také jsme zjistili, že periferní aplikace LPI u myší vede k mechanické přecitlivělosti v závislosti na dávce, aniž by byl vyvolaný zánět, demyelinizace nebo extravazace.

Tyto výsledky naznačují možnou roli LPI-GPR55 signalní dráhy v rakovinou vyvolané bolesti. Blokování GPR55 signální dráhy specifickými ligandy by tedy mohlo sloužit jako potenciální terapeutická strategie k léčbě onkologické bolesti.

ABSTRACT

Charles University in Prague

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

Candidate: **Dagmar Škoricová**

Supervisors: **Prof. Dr. Rohini Kuner**

Doc. PharmDr. Petr Pávek, Ph.D.

Title of diploma thesis: **Dissecting the role of GPR55 agonist lysophosphatidylinositol in mechanically evoked pain**

Recent studies have suggested that the orphan receptor GPR55 (G-protein coupled receptor 55) is possibly a novel cannabinoid receptor activated by multiple different cannabinoid ligands and also by the lysophospholipid L-α-lysophosphatidylinositol (LPI).

GPR55 is reported to be expressed on sensory neurons and mice lacking GPR55 were shown to have reduced mechanical hypersensitivity in inflammatory and neuropathic pain models, suggesting a pronociceptive role of GPR55. LPI was found to be secreted in large quantities from varieties of cancer tissue. These facts led us to speculate that LPI released from cancer cells could sensitize nociceptors and thereby lead to cancer-induced pain.

The aim of the study was to investigate the role of LPI in cancer induced, mechanically evoked pain and its possible underlying mechanism. We found that the dorsal root ganglion neurons are directly activated by LPI *in vitro*. We also found that LPI injected in the periphery leads to a mechanical hypersensitivity in mice in a dose dependent manner without eliciting any inflammation, demyelination or extravasation.

These results indicate a possible role of LPI-GPR55 signaling pathway in cancer induced pain. Blocking of GPR55 signaling with specific ligands might therefore serve as a potential therapeutic strategy to treat cancer induced pain.

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1 INTRODUCTION

The sensation of pain is one of the crucial physiological functions of the nervous system as it is a response of an organism to actual or potential harm in order to avoid tissue damage. However, sustained or chronic pain can result in secondary symptoms such as anxiety or depression associated with decrease of the quality of life. This kind of pain no longer has a protective role and should be considered a disease itself rather than just a symptom.

Demographical studies concerning pain prevalence vary widely, mainly depending on the methodology and also because pain is a subjective phenomenon with no available standard clinical tool, so the studies rely on self-reported measures of pain. All studies, however, leave no question that persisting pain occurs in high rates in people of all ages.

In general, 33 % of European population has already experienced severe pain in some episode of the life, whereas 18 % of European people reports ongoing middle to severe chronic pain condition. The prevalence of pain at the time of cancer diagnosis is estimated to be approximately 50 % in the early stage, increasing to 75 % at advanced stages, while cancer patients experience pain as the result not only of the disease, but also of its treatment, surgery, diagnosis or unrelated causes (www.iasp-pain.com).

The impact and behavioral course of painful conditions varies across individuals, whereas the common disruptive pattern concerning psychological distress, depression or anxiety disorders interfere with their normal activity and cause severe social problems.

2 PAIN

There are various definitions of pain, whereas the most accurate seems to be the definition of The International Association for the Study of Pain (IASP), which defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage or both (www.iasp-pain.org).

While nociceptive pain is a protective physiological component of the normal nervous system, pathological pain typically results from damage to the nervous system (neuropathic pain) or its abnormal functioning (dysfunctional pain). Neuropathic pain also commonly occurs as a secondary symptom in diseases, such as diabetes, cancer or herpes zoster infection and it is often characterized as stimulus-independent persistent pain or abnormal sensory sensation of pain (Basbaum 1999). A special class of pain, inflammatory pain, is associated with a tissue damage and infiltration of cells contributing to immune response (Woolf) (Figure 2.1).

In the cancer population, neuropathic pain often results from nerve compression, direct neoplastic invasion of the peripheral nerves or spinal cord, or the neuropathy is related to the treatment (Farrar and Portenoy 2001), but it can also be caused by ischemia or proteolysis that injures the sensory and sympathetic nerve fibers (Usunoff et al. 2006). Moreover, tumors produce a variety of factors that sensitize or directly excite nociceptors, causing pain sensation (Mantyh et al. 2002).

Signs and symptoms of pathological pain differ in disease and patient, but they share certain clinical characteristics: continuous pain, usually of a dull and burning character, paroxysmal pain (shooting, lancinating), lowered pain threshold to various mechanical or thermal, noxious or non-noxious stimuli (Usunoff et al. 2006).

Somatic sensibility is mediated by different types of receptors distributed throughout the body. Each of these receptors is morphologically and molecularly specialized to selectively respond to the specific type of stimuli. Periphery receptors recognize discriminative touch (size, shape, and texture of objects), proprioception

(position and movement of the body), nociception (tissue damage or chemical irritation), and temperature sense (warmth and cold) (Kandel et al. 2000).

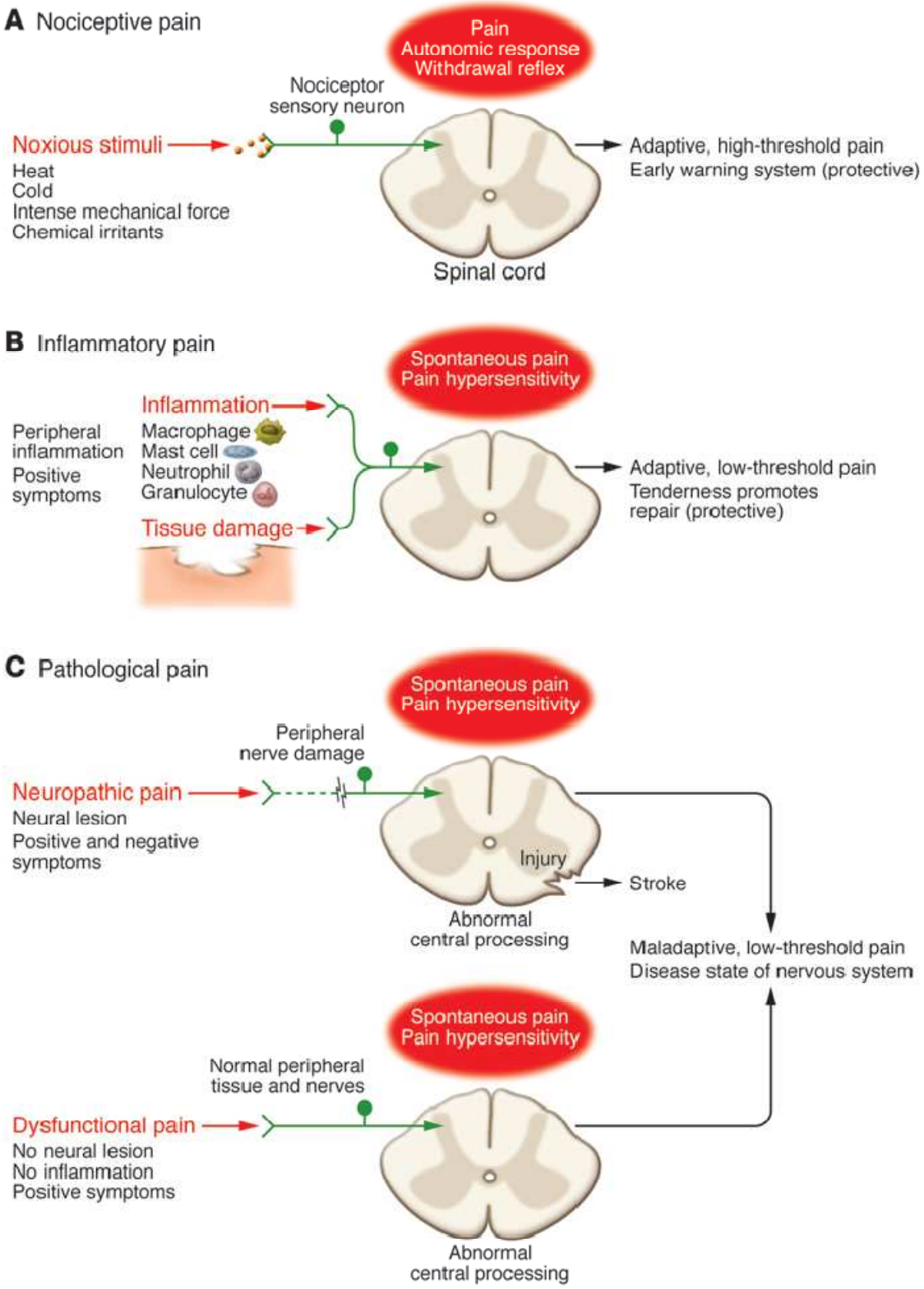


Figure 2.1. Pain classification (Woolf) (A) Nociceptive pain with protective function. (B) Inflammatory pain with contribution of immune system. (C) Pathological pain associated with nerve damage or abnormal functioning.

Each of the modalities is mediated by different receptors and pathways to the brain. However, all these receptors are part of the periphery terminals of sensory neurons, which cell bodies are located in the dorsal root ganglia for the body and trigeminal ganglia for the facial area (Kandel et al. 2000).

2.1 Nociceptors and nociception

Nociceptors, a term first mentioned by Sherrington in 1906, are the primary sensory neurons that respond selectively to stimuli that can cause tissue damage (Sherrington 1906). Nociceptors have four major functional components, the peripheral terminal, the axon, the cell body and the central terminal (Woolf and Ma 2007) (Figure 2.2.). Nociceptors, together with other sensory neurons belong to the family of pseudo-unipolar cells with single axon that bifurcates into two branches. One projecting to the central nervous system and one projecting to the periphery as free nerve endings, where it transduces external stimuli and initiates action potentials. Nociceptive primary afferent axons terminate in the dorsal horn of the spinal cord, which is the site of the first synapse on nociceptive second order neurons in spinothalamic and spinobrachial ascending pathways conveying to the brain sensory information underlying conscious perception of pain (Kandel et al. 2000).

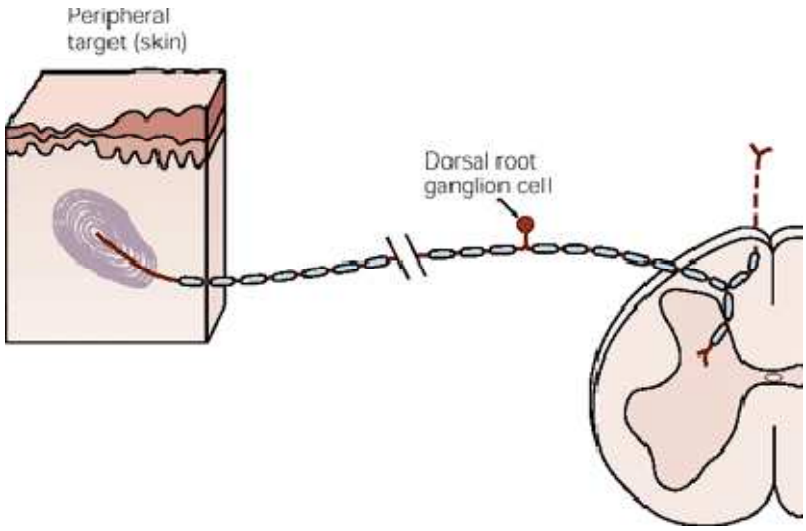


Figure 2.2. The morphology of a dorsal root ganglion cell (Kandel et al. 2000)

Peripheral somatosensory neurons contain several different types of axons that carry sensory information, which divides cutaneous nociceptors into two major classes (Kuner 2003). Medium diameter myelinated Aδ mechanoreceptors, whose stimulation leads to fast, pricking pain and unmyelinated C-nociceptors, whose stimulation evokes sensation of slow, burning or dull pain (Basbaum et al. 2009).

Aδ fibers are high-threshold nociceptors that respond to noxious mechanical stimuli, whereas Aα involved in proprioception and Aβ cutaneous mechanoreceptors, normally do not conduct noxious stimuli. Aδ fibers respond to painful stimuli caused by sharp objects that penetrate, squeeze, or pinch the skin (Meyer et al. 2006) and they are further divided according to their differential responsiveness to intense heat and their sensitization ability (Julius and Basbaum 2001).

C fibers are slowly conducting afferents present in the majority of sensory neurons in peripheral nervous system, but not all of them are involved in nociception. Some also mediate pleasant touch or respond to stroking of the hairy skin (Olausson et al. 2008). Most C-fiber nociceptors are polymodal, responding nonselectively to noxious thermal, mechanical and chemical stimuli (Julius and Basbaum 2001).

Silent nociceptors are a special class of unmyelinated afferents, which are heat responsive, but normally mechanically insensitive, however, they become mechanically sensitive only when sensitized by tissue injury (Schmidt et al. 1995).

Primary afferents release a variety of chemical mediators, but the principal excitatory neurotransmitter is amino acid glutamate. Glutamate evokes fast synaptic potentials in dorsal horn neurons by activating several types of postsynaptic glutamate receptors, such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, N-methyl-D-aspartase (NMDA) receptors and metabotropic glutamate receptors (mGluRs) (Kuner 2003). The primary afferent fibers of nociceptive neurons also elicit slow excitatory postsynaptic potentials in dorsal horn neurons by releasing various peptide neurotransmitters, such as substance P and calcitonin gene-related peptide (CGRP). They are involved in the response to intense stimulation of peripheral nerves as they contribute to the spread of oedema by their vasodilatating effect and to sensitization of nociceptors by releasing of histamine from mast cells. Moreover,

neuroactive peptides also appear to enhance and prolong the actions of glutamate and their levels are significantly increased in persistent pain conditions, suggesting that they play an important role in developing of neuropathic pain states (Kandel et al. 2000).

2.2 Sensitization

In pathological situations, repeated activation of nociceptors can lead to various states of abnormal pain called sensitization (Nestler et al. 2009).

Sensitization of nociceptors after injury or inflammation is induced by chemical mediators released by the damaged cells and tissues, such as prostaglandins, leukotrienes, cytokines, chemokines, growth factors, bradykinin, histamine, acetylcholine, serotonin, substance P and others. They all act to decrease the threshold for activation of nociceptors which results in excessive response to noxious stimuli – hyperalgesia. Primary hyperalgesia occurs in the damaged area, whereas secondary hyperalgesia occurs when also surrounding area becomes sensitive (Kuner 2003). Some substances, however, directly activate nociceptors leading to the sensation of pain in response to normally innocuous stimuli, referred to as allodynia (Kandel et al. 2000).

Heightened sensitivity presumably serves the adaptive purpose of leading an organism to protection of the injured area in the short term, but it is a major cause of clinically significant pain if it persists. Sensitization of primary nociceptors may lead to long-term changes in second-order neurons within the dorsal horn called central sensitization. It is a form of excessive excitatory synaptic responses in nociceptive neurons, which leads to an increased gain of the pain transmission system and pain hypersensitivity (Nestler et al. 2009).

The combination of peripheral and central sensitization underlies neuropathic pain, resulting in chronic allodynia, hyperalgesia, and spontaneous pain (Nestler et al. 2009).

2.3 G-protein-coupled receptors (GPCRs)

Primary afferent somatosensory neurons possess a rich diversity of ligand-gated ionotropic, metabotropic and tyrosine kinase receptors. Many mediators produced during pain and inflammation, such as bradykinin, serotonin and prostaglandins, act via G-protein-coupled receptors (GPCRs). GPCRs are the plasma membrane receptors with seven transmembrane domains and a cytoplasmic domain. The cytoplasmic domain interacts with an intracellular heterotrimeric guanine nucleotide regulatory protein (G-protein) consisting of three subunits (α, β and γ). Subsequent biochemical response depends on the type of α subunit that is activated. $G_{s/q}$ stimulates, whereas $G_{i/o}$ inhibits the activity of various enzymes (adenylate cyclases, guanylate cyclases, phospholipases, Ras etc..) leading to increase of level of diffusible second messengers, such as cyclic adenosine monophosphate (cAMP), diacylglycerol or inositol polyphosphate. G-proteins can also directly influence ion channels. The subsequent biochemical cascade leads to phosphorylation of cell proteins or mobilization of Ca^{2+} ions from intracellular stores (Strader et al. 1995; Kandel et al. 2000) (Figure 2.3.).

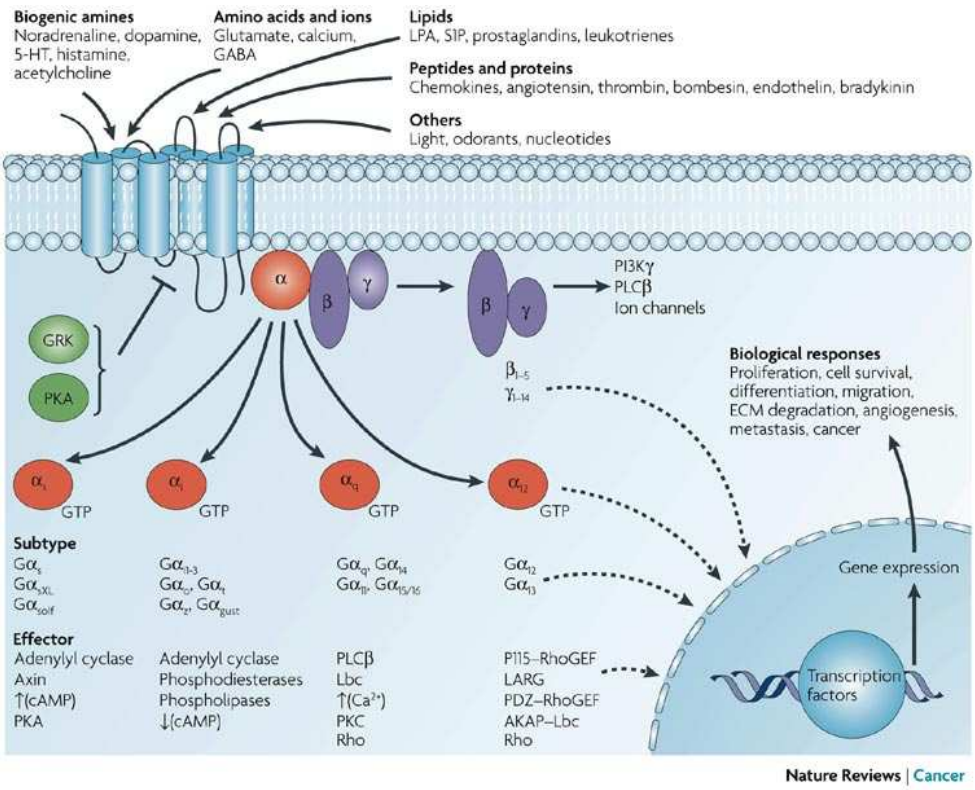


Figure 2.3. Diversity of G-protein coupled receptor signal transduction (Dorsam and Gutkind 2007)

2.3.1 Cannabinoid receptors

Two metabotropic cannabinoid receptors, CB1 and CB2 have been identified since 1988, as the receptors through which cannabinoids (endogenous, plant or synthetic ligands) exert their effect (Devane et al. 1988). They are both $G_{i/o}$-coupled and their activation leads to inhibition of cAMP production via adenylate cyclase and activation of mitogen-activated protein kinase (MAPK) (extracellular-regulated protein kinase-ERK). This signal transduction pathway leads to phosphorylation of critical amino acid residues on the intracellular surfaces (Ryberg et al. 2007).

CB1 receptors are predominantly expressed in the CNS and by primary afferent neurons in peripheral nervous system and they are mainly involved in attenuation of synaptic transmission (Howlett 2002). Distribution of CB2 receptor is restricted to the cells of the immune system including glia, with particularly high levels in B cells and natural killer cells (Galiegue et al. 1995). Therefore, CB2 receptors are implicated in the regulation of inflammatory reactions and immune response as they are known to modulate cytokine release (Pertwee et al.). They are widely distributed along the pain-regulatory circuits, which makes them potential target for therapy of different pain states.

2.3.1.1 Cannabinoids

Cannabinoids have significant antinociceptive efficacy, which has been used for centuries for therapy of pain (Walker and Huang 2002), but the problem of physical and psychological side effects in the same dose range as that for analgesia limits their use as analgetics (Malan et al. 2003).

Cannabinoids are a chemically heterogeneous group divided into three categories: the endocannabinoids, the phytocannabinoids and the synthetic compounds with cannabinoid-like activity. Cannabis sativa contains more than 60 phytocannabinoids, whereas the most abundant constituents include Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN). THC is thought to mediate its psychoactive and addictive properties mainly through neuronal CB1 receptors (Hall and Solowij 1998), whereas CBD and CBN are not psychoactive and act mostly on CB2 receptors (Ross 2009). The antinociceptive effect of cannabinoids is mediated by suppression of

nociceptive transmission observed in behavioral studies using thermal, mechanical and chemical noxious stimulation (Walker and Huang 2002).

The identification of cannabinoid receptors expressed in mammalian tissues was followed by an intensive search for their endogenous ligands, which revealed the family of natural cannabinoid agonists – endocannabinoids (Devane et al. 1992; Sugiura et al. 1995). Their physiological role is still not well understood, but experiments with cannabinoid receptors deficient mice (Ledent et al. 1999; Zimmer et al. 1999; Buckley et al. 2000) suggest that their function is more modulatory rather than primary physiological. The processes influenced by endocannabinoids include cognition and memory, motor coordination, temperature homeostasis and sleep. The endocannabinoid system is also involved in the pathophysiology of obesity, osteoporosis, addiction, mental illness, neuropathic and inflammatory pain, cardiovascular disorders and liver diseases (Ross 2009).

There are recently known a couple of endogenous ligands for CB receptors, whereas the most studied are anandamide (N-arachidonoylethanolamine, AEA) and 2-arachidonoyl glycerol (2-AG) (Sugiura and Waku 2002).

2.3.2 GPR55

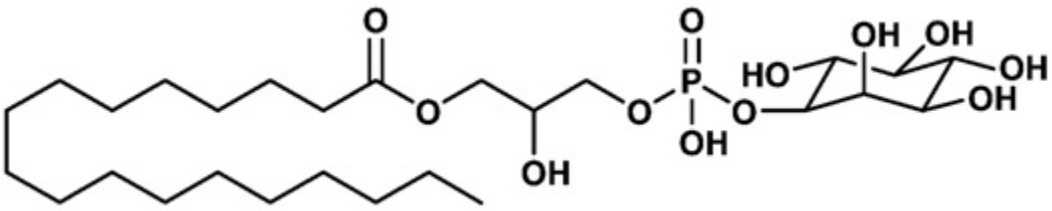
Studies performed with CB1 and CB2 deficient mice revealed circumstantial evidence for the existence of further cannabinoid receptors or subtypes (Howlett et al. 2002; Begg et al. 2005; Mackie and Stella 2006). GPR55 showed the ability to interact and to be modulated by a variety of endogenous and exogenous cannabinoid ligands (Mackie and Stella 2006) and it has recently been classified as a putative third cannabinoid receptor (Ryberg et al. 2007). However, there are still some questions concerning this assignment and GPR55 pharmacology remains controversial.

GPR55 is a seven transmembrane GPCR highly expressed in large-diameter DRG neurons (Lauckner et al. 2008), brain, lymphoid organs, immune cells, endothelial cells, osteoclastes and osteoblastes, which suggests that GPR55 plays a role in these tissues known to respond also to cannabinoids (Sharir and Abood). However, exact physiological or pathophysiological significance of this receptor remains elusive, but

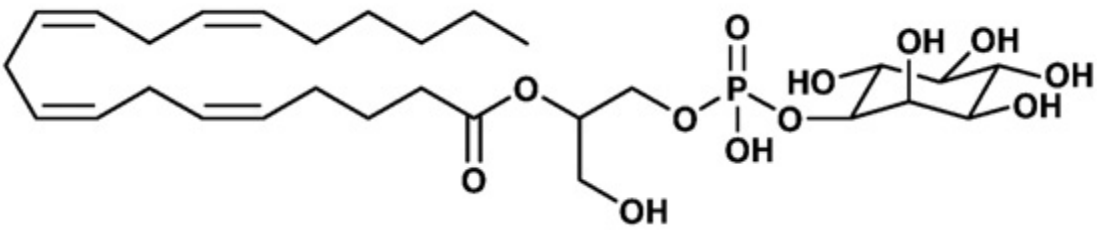
regulation of both inflammatory and neuropathic pain seems to be one of its potential role, while experiments with GPR55 deficient mice revealed that these mice failed to develop mechanical hyperalgesia in models of inflammatory and neuropathic pain. Moreover, the levels of anti-inflammatory cytokines have increased as compared with wild-type animals (Staton et al. 2008).

2.3.2.1 LPI (L-α-lysophosphatidylinositol)

LPI is a bioactive lipid belonging to a family of lysophospholipids, which are molecularly diverse structures composed of various lengths of acyl chains containing saturated, monosaturated or polyunsaturated fatty acid at either *sn*-1 or *sn*-2 position. 2-arachidonoyl-*sn*-glycero-3-phosphoinositol seems to have the greatest biological activity towards GPR55 compared to LPI species containing other fatty acyl groups (Oka et al. 2009) (Figure 2.4.).



Lysophosphatidylinositol



2-arachidonoyl-*sn*-glycero-3-phosphoinositol

Figure 2.4. Chemical structures of lysophosphatidylinositol and 2-arachidonoyl-*sn*-glycero-3-phosphoinositol, GPR55 lysophospholipid agonists

LPI induces stimulation of [^{35}S]GTPγS-binding to G-proteins (agonist-induced GDP-GTP exchange as an indicator of receptor activation), indicating that it exerts its effects via a G-protein-coupled apparatus. LPI was recently reported to be a GPR55 endogenous ligand (Oka et al. 2007).

Stimulation of GPR55 by LPI is known to activate $G_{\alpha 12}$, $G_{\alpha 13}$ or G_q proteins and to induce rapid phosphorylation of the extracellular signal-regulated kinase (ERK 1/2) in transiently or stably GPR55-expressing HEK293 cells (Henstridge et al.; Oka et al. 2007). Furthermore, LPI increases intracellular free calcium concentration possibly in the tissue-dependant manner, which as a consequence activates NFAT. (Henstridge et al.; Lauckner et al. 2008; Henstridge et al. 2009). NFAT as a key molecular target for GPR55 then binds DNA and regulates the transcriptional activity of a number of genes (Im and Rao 2004).

LPI is one of the key signaling intermediates that controls diverse aspects of cellular functions (Henstridge et al. 2009), but except for its physiological functions it was also found to contribute to cell growth of certain tumors. This mitogenic activity of LPI was demonstrated by several investigators (Falasca and Corda 1994; Falasca et al. 1998), finding that it is mediated by GPR55 receptor expressed in prostate and ovarian cancer cell line (Pineiro et al.). Blocking of GPR55 or its downregulation inhibits tumor cell proliferation, which indicates that LPI could constitute a novel cancer biomarker and GPR55 a potential therapeutic target for cancer treatment (Andradas et al.).

3 AIM OF THE WORK

As this study is a part of a bigger project dissecting the role of LPI-GPR55 pathway in peripheral sensitization in pain states, the primary focus of this thesis is:

1. To investigate whether bioactive phospholipid LPI can directly activate neurons of the dorsal root ganglion.
2. To study if peripheral application of LPI leads to mechanical hypersensitivity in mice.
3. To determine whether the mechanical hypersensitivity caused by LPI is mediated by
    a. inflammation
    b. extravasation
    c. demyelination

4 MATERIALS

4.1 Animals

All animals in this study were used according to the ethical guidelines of the local governing body. Animals were maintained with food and water ad libitum under 12-hour light/dark cycle. All behavioral experiments were done in 2-3 months old, awake, unrestrained, age-matched, male or female mice (C57/Bl6).

4.2 Materials

Butterfly needle *(BD microlance; BD Bisciences, GE)*

Carbon dioxide tank *(Air Liquide Medical GmbH; Düsseldorf, GE)*

Coverslips *(Medite GmbH; Brugdorf, GE)*

Falcon tubes 15 ml, 50 ml *(Sarstedt AG & Co.; Nümbrecht, GE)*

Glass pipettes *(Axon Labortechnik GmbH; Kaiserslautern, GE)*

Hamilton syringe *(Hamilton Bonaduz AG; Bonaduz, Switzerland)*

Insulin syringe *(BD finulane; BD Bisciences, GE)*

Microscope slides *(NeoLab Migge Laborbedarf-Vertriebs GmbH; Heidelberg, GE)*

Microtube 1.5 ml *(Sarstedt AG & Co.; Nümbrecht, GE)*

Mini trans-blot cell and Electrophoresis module (Mini-PROTEAN Tetra system) (*Bio-Rad Laboratories GmbH; München, GE)*

Nitrocellulose transfer membrane *(Whatman GmbH; Dassel, GE)*

Pipette *(Gilson, Inc.; Middleton, WI, USA)*

Pipette boy – neoAccupette *(Wager & Munz GmbH; München, GE)*

Pipette tips *(Greiner bio-one GmbH; Frickenhausen, GE)*

Radiographic cassette *(Dr.Goos-Suprema GmbH; Heidelberg, GE)*

Serological pipette 5 ml, 10 ml, 25 ml *(Sarstedt AG & Co.; Nümbrecht, GE)*

Suture material Marlin® *(Catgut GmbH; Markeneukirchen, GE)*

Tissue culture dishes *(Becton Dickinson Labware; Le Pon de Claix, France)*

Tissue freezing medium *(R. Jung GmbH; Nußloch, GE)*

Von Frey Hairs - with grid *(Ugo Basile; Italy)*

X-Ray film *(FujiFilm Europe GmbH; Düsseldorf, GE)*

4.3 Instruments

Centrifuge *(Kendro Laboratory Products GmbH; Osterode, GE)*

CO_2 Incubator *(Thermo Fisher Scientific Inc.; Egelsbach, GE)*

Freezer -80°C *(Haraeus Sepatech GmbH; Osterode, GE)*

Homogenizer *(PRO Scientific Inc.; Oxford, CT, USA)*

Hood *(Thermo Electron LED GmbH; Langenselbold, GE)*

Leica cryotome CM 3050 S *(Leica Microsystems GmbH; Wetzlar, GE)*

Microscope Leica DM LS2 *(Leica Microsystems GmbH; Wetzlar, GE)*

Nanodrop *(Peqlab Biotechnologie GmbH; Erlangen; GE)*

Plantar Test - Hargreaves Apparatus *(Ugo Basile; Italy)*

Power supply *(Buddeberg GmbH; Mannheim, GE)*

Surgical tools (forceps, scissors, scalpels) *(Fine Science Tools GmbH, Heidelberg, GE)*

Syringes *(BD Syringe; BD Bisciences, GE)*

Syringe needle *(BD microlance; BD Bisciences, GE)*

Thermomixer *(Eppendorf AG; Hamburg, GE)*

Vortex machine *(IKA® Werke GmbH & Co. KG; Staufen, GE)*

X-Ray film processor *(PROTEC Medizintechnik GmbH & Co. KG; Oberstenfeld, GE)*

4.4 Chemicals

All used chemicals were in the highest purity.

2-mercaptoethanol *(Carl Roth GmbH & Co. KG; Karlsruhe, GE)*

2-propanol or isopropanol *(Merck KGaA; Darmstadt, GE)*

Acetic acid anhydrous *(Merck KGaA; Darmstadt, GE)*

Acryl-bisacrylamide mix – Rotiphorese Gel 30 *(Carl Roth GmbH & Co. KG; Karlsruhe, GE)*

APS *(Grüssing GmbH; Filsum, GE)*

Bromophenol Blue *(Waldeck GmbH & Co. KG; Münster, GE)*

BSA - Albumin fraktion V *(Carl Roth GmbH & Co. KG; Karlsruhe, GE)*

CFA *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

D(+)- Saccharose *(Carl Roth GmbH & Co. KG; Karlsruhe, GE)*

DTT *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

EDTA *(AppliChem GmbH; Darmstadt, GE)*

Ethanol 99% *(Merck KGaA; Darmstadt, GE)*

Evans blue *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

Fast green dye *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

Formamide *(Mallinckrodt Baker, Inc; Phillipsburg, NJ, USA)*

FBS *(Invitrogen GmbH; Darmstadt, GE)*

Formalin, 10% Neutral Buffered - 4% PFA *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

Glycerol anhydrous *(AppliChem GmbH; Darmstadt, GE)*

Glycin *(AppliChem GmbH; Darmstadt, GE)*

H_2O_2 30% *(Carl Roth GmbH & Co. KG; Karlsruhe, GE)*

HCl fuming 37% *(Merck KGaA; Darmstadt, GE)*

Histamine *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

Horse serum *(Invitrogen GmbH; Darmstadt, GE)*

Chloroform *(VWR International GmbH; Darmstadt, GE)*

Isoflurane *(Baxter Deutschland GmbH; Heidelberg, GE)*

KCl *(AppliChem GmbH; Darmstadt, GE)*

KH_2PO_4 *(Grüssing GmbH; Filsum, GE)*

LPI *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

Methanol *(Merck KGaA; Darmstadt, GE)*

Molecular weight marker – Protein ladder #1811 *(Fermentas GmbH; St. Leon-Rot, GE)*

Mowiol 4-88 *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

$Na_2HPO_4.2H_2O$ *(AppliChem GmbH; Darmstadt, GE)*

NaCl *(VWR International GmbH; Darmstadt, GE)*

Nerve growth factor *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

NFDM – Milk powder *(Carl Roth GmbH & Co. KG; Karlsruhe, GE)*

Nonidet P40 *(Fluka Chemie AG; Buchs, Schwitzerland)*

Percoll *(GE Healthcare Bio – Sciences AB; Uppsala, Sweden)*

Phosphatase inhibitor *(Roche Diagnostics GmbH; Mannheim, GE)*

Poly-L-Lysine 0.1% *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

Ponceau S *(AppliChem GmbH; Darmstadt, GE)*

PP2 *(Merck KgaA; Darmstadt, GE)*

Protease inhibitor *(Roche Diagnostics GmbH; Mannheim, GE)*

SDS *(Serva Electrophoresis GmbH; Heidelberg, GE)*

Sodium borate *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

Sodium desossicolate *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

Sodium orthovanadate *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

TEMED *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

Toluidine blue *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

Tris base *(Carl Roth GmbH & Co. KG; Karlsruhe, GE)*

Triton X-100 *(Merck KGaA; Darmstadt, GE)*

Trypsin inhibitor *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

Tween 20 *(Carl Roth GmbH & Co. KG; Karlsruhe, GE)*

Xylene *(Grüssing GmbH, GE)*

4.5 Enzymes

Collagenase *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

DNAse *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

Trypsin *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

4.6 Mediums

DMEM *(Gibco, Invitrogen GmbH; Darmstadt, GE)*

DPBS 10x *(Gibco, Invitrogen GmbH; Darmstadt, GE)*

F12 *(Gibco, Invitrogen GmbH; Darmstadt, GE)*

4.7 Antibiotics

Penicillin Streptomycin *(Invitrogen GmbH; Darmstadt, GE)*

4.8 Antibodies

Primary: P44/42 MAPK (ERK 1/2) rabbit Ab *(Cell Signaling Technology, Inc.; Frankfurt am Main, GE)*

Phospho-P44/42 MAPK (p-ERK 1/2) rabbit Ab *(Cell Signaling Technology, Inc.; Frankfurt am Main, GE)*

Biotin Rat anti-mouse Ly-6G and Ly-6C clone RB6-8C5 (Gr-1) *(BD Biosciences, Franklin Lakes, NJ, USA)*

Rat IgG Biotinylated Ab *(Vector Laboratories, Inc.; Burlingame, CA, USA)*

Secondary: Anti-Rabbit IgG (whole molecule)-Peroxidase *(Sigma-Aldrich Chemie GmbH; Steinheim, GE)*

4.9 Kits

ABC Kit – Vercastain, Peroxidase standard *(Vector Laboratories, Inc.; Burlingame, CA, USA)*

DAB Kit – Peroxidase substrate kit *(Vector Laboratories, Inc.; Burlingame, CA, USA)*

Immobilon Western, Chemimuminescent HRP Substrate *(Millipore Corporation; Billerica, USA)*

4.10 Buffers

Table 1. Buffers for western blot

Leammli buffer 2x:	4% SDS
	2% 2-mercaptoethanol or 200 mM DTT
	20% glycerol
	0.004% bromophenol blue
	0.5 M Tris HCl pH 6.8
	add ddH_2O

Lysis buffer (RIPA buffer):	50 mM Tris HCl pH 7.4
	150 mM NaCl
	1% NP-40
	0.5% sodium desossicolate
	0.1% SDS
	add ddH_2O
Just before use add:	1 protease inhibitor coctail tablet
	1 phosphatase inhibitor coctail tablet
	2 mM sodium orthovanadate

Running buffer:	25 mM Tris HCl pH 8.3
	190 mM glycine
	0.1% SDS
	add ddH_2O

TBST:	0.1% Tween 20
	add TBS

Transfer buffer:	25 mM Tris HCl pH 8.3

	190 mM glycine
	0.1% SDS
	20% methanol
	add ddH_2O

Mild stripping buffer:	200 mM glycine
	0.1% SDS
	1% Tween 20
	adjust to pH 2.2 with HCl
	add ddH_2O

Table 2. Buffers for staining

PBS/ H_2O_2/ CH_3OH:	1% H_2O_2
	add PBS + 99% methanol (1:1)

PBS/ NHS:	10% NHS
	add PBS
PBST:	0.1% Triton
	add PBS

4.11 Solutions

Table 1. Gels for western blot

Separating gel 10%:	10% acryl-bisacrylamide mix
	400 mM HCl pH 8.8
	0.1% SDS
	0.1% APS
	0.04% TEMED
	add ddH_2O

Stacking gel 4 %:	4% acryl-bisacrylamide mix
	200 mM HCl pH 6.8
	0.1% SDS
	0.1% APS
	0.1% TEMED
	add ddH_2O

Table 2. Stock solutions

TBS 10x:	24.23 g Tris
	0.06 g NaCl
	mix in 800 ml ddH_2O
	adjust to pH 7.6 with HCl
	add 1000 ml ddH_2O

PBS 10x:	80.0 g NaCl
	2.0 g KCl
	18 g $Na_2HPO_4.2H_2O$
	2.4 g KH_2PO_4
	adjust to pH 7.4 with HCl
	add up 1000 ml with ddH_2O

Table 3. Stock solutions for western blot

BSA (or milk) 5% solution:	5% BSA (or NFDM)
	add TBST

Ponceau Red solution:	0.2% Ponceau S
	5% acetic acid
	add ddH_2O

Table 4. Solution for cell culture

Enzyme solution:	0.25 mg/ml Trypsin
	1 mg/ml Collagenase
	0.2 mg/ml DNAse
	add F12 + P/S

F12 + 10% FBS + AraC:	10% FBS
	5 µM AraC
	10 ng/ml NGF
	add F12 + P/S

Percoll solutions:	
SIP	9 v/v (9 ml) Percoll
	1 v/v (1 ml) PBS 10x
Percoll 35% (5 ml):	1.75 ml SIP
	3.25 ml F12
Percoll 25% (5 ml):	1.25 ml SIP
	3.75 ml DMEM

Table 5. Solution for staining

Mowiol solution:	2.4 g Mowiol 4-88
	6.0 g Glycerin
	6.0 ml ddH_2O
	12.0 ml 0.2 M Tris-HCl pH 8.5

4.12 Software

Adobe Photoshop CS2

Endnote X1

Graph prim 5

ImageJ

Microsoft Office

5 METHODS

5.1 Von Frey test

For mechanical testing, the mice were placed individually in a small plastic cage with an open wire mesh bottom. The mice were acclimatized to the setup before testing and were unaware of the testing. Mechanical sensitivity was measured by applying punctuate pressure using 0.07g Von Frey filaments. The filaments were applied perpendicularly to the plantar surface in the middle of the mouse's hind paw with an upward force just sufficient to bend the microfilament. A positive response was considered a paw withdrawal before the filament bending and we measured frequency of response out of five applications.

5.2 DRG culture

5.2.1 Isolation of dorsal root ganglia

After anesthetizing the mice with CO_2, the animals were decapitated and vertebral column was surgically exposed. 30-40 dorsal root ganglia were quickly dissected and placed into ice cold F12+GlutaMax medium containing 2 % Penicillin and Streptomycin (P/S). Subsequently, surrounding membranes, axons and meningeal residues were separated from actual cell bodies.

5.2.2 Cell culture preparation

The clean DRGs were digested with 1 ml of the enzyme solution for 30 minutes at 37°C in agitation (thermomixer 14000 rpm) and mechanically dissociated with the previously coated pipette subsequently. The digestion was stopped by adding 120 µl of Trypsin inhibitor and 100 µl of 10% FBS. The suspension was centrifuged for 3 minutes at 1000 rpm.

After centrifugation, the supernatant was removed and cells were resuspended into 2ml F12+Glu+P/S medium with 10% FBS. The cell suspension was then carefully loaded on Percoll gradient (2.5 ml of 25% the upper phase and 1.5 ml of 35% the lower phase)

and centrifuged for 15 minutes at 1000g. Percoll gradient creates the density gradient that separates the cells on the basis of their size and density. The lower part of the supernatant (approximately 1.5 ml) and the pellet was kept and washed 2 times, first time with 13.5 ml F12+GlutaMax+P/S and 10% FBS and after 10 minute centrifugation (500g) with 9 ml F12+GlutaMax+P/S and 10% FBS, then centrifuged for 8 minutes (400g). The pellet was resuspended in 320 µl F12+GlutaMax+P/S and 10% FBS and distributed to the wells previously coated with poly-L-lysine. Firstly, 60 µl of the cell solution was placed in the middle of each plate and after 10 minutes the total volume up to 200 µl of F12+GlutaMax+P/S and 10% FBS was added.

The cells were grown in the medium for 3 days at 37°C in a atmosphere of 5 % CO_2. The vitality of the culture was checked every day and the medium was replaced by the fresh one with additional NGF (neural cells growth factors) and AraC.

5.2.3 Treatment of the cells

On the fourth day, the cells were refed with F12+GlutaMax+P/S culture medium deprived of serum. This starvation serves as a pre-treatment of the cells in order to lower the metabolism resulting in the increased efficacy of the response following the treatment. After 2 hours, the cells were treated with 2 ml of 3 µM LPI for 15 minutes.

5.3 Western blot

Western blot is a method widely used to detect specific proteins, which are separated in denatured state according to their molecular weight using SDS polyacrylamide gel electrophoresis (SDS-PAGE). The proteins are subsequently transferred onto nitrocellulose or PVDF membrane and detected by antibodies specific to the target protein.

5.3.1 Samples preparation

100 µl of complete lysis buffer together with protease and phosphatase inhibitors were added into the cell solution right after treatment. The DRG samples were then homogenized by sonicator 3 times each for 3 seconds and left for 30 minutes in ice,

occasionally vortexed. After complete cell lysis, the homogenate was clarified by centrifugation at 13000 rpm for 15 minutes at 4°C. The supernatant, corresponding to the cytosolic fraction was collected and the protein concentration was determined by Nanodrop by 260/280 nm spectrophotometry.

Before the loading, protein samples were boiled for 5 minutes together with Leammli buffer, which caused denaturation of proteins. Denaturation achieves the linear state of proteins, so that they no longer have any secondary, tertiary or quartery structure and can be easily separated according to their size. After the denaturation, the samples were centrifuged for 1 minute at 11000 rpm.

5.3.2 SDS-PAGE gel preparation

SDS-PAGE gels separate proteins according to their size in the presence of electric current. SDS is a denaturing detergent that binds to the proteins' positive charged amino acids and neutralizes them; therefore all proteins become negatively charged. Thus they migrate towards the positively charged electrode when placed in an electric field, whereas smaller molecules move faster than bigger and proteins become separated according to their size.

Separation of proteins was performed by SDS-PAGE using 1 mm thick gel sandwich set out of 2 discontinues phases – separation gel and stacking gel. The purpose of using two different gels is to concentrate isolated proteins and place them to the boundary-line so they all enter separation gel at the same time.

To separate the proteins, 10% separating gel was used consisting of 1.5 ml of 25% separation buffer, 2.0 ml of 30% acrylamide/bisacrylamide mixture, 2.4 ml of ddH_2O, 100 µl of APS and 5 µl of TEMED. The polymerization of acrylamide and N,N-methylenebisacrylamide is catalyzed by addition of ammonium persulfate (APS) and TEMED, therefore they were added just before casting of the gel. The gel was allowed to polymerize at room temperature under isopropanol level, which ensures smooth surface of the gel. After complete consolidation, the isopropanol was removed and with inserted comb, 4% stacking gel was casted onto polymerized separating gel and

was let polymerize. The gel consisted of 0.88 ml of 25% stacking buffer, 0.56 ml of 30% acrylamide/bisacrylamide mixture, 2.01 ml of ddH_2O, 50 µl of APS and 5 µl of TEMED.

5.3.3 SDS-PAGE gel electrophoresis

After complete polymerization of the stacking gel, gels were assembled in the vertical electrophoresis chamber with running buffer. The comb was removed from the stacking gel and samples were loaded into the gel wells in desired volume together with 10 µl of protein molecular weight marker. The constant voltage of 120 V was used to conduct electrophoresis until the blue dye migrated to the bottom of the gel.

The separation of molecules in a gel depends on the percentage of the gel, which is determined by the total amount of acrylamide and the amount of cross-linking agent (N,N-methylenebisacrylamide) present in the gel. The pore size increases with less amount of acrylamide. Therefore for larger protein lower percentage of acrylamide should be used, whereas for smaller protein higher percentage of acrylamide is preferred.

5.3.4 Protein electrotransfer

After protein separation by SDS-PAGE electrophoresis, proteins were transferred from the gel onto previously wetted nitrocellulose membrane in order to make them accessible to detection. The membrane was placed on top of the gel and sandwiched by filter papers and sponges ensuring no air bubbles between the layers. This blotting sandwich consisting of sponge-paper-gel-membrane-paper-sponge was assembled in the transfer chamber in the correct orientation, so that the membrane is on the side close to the anode. Transfer was performed at constant amperage of 300 mA for 2 hours in the presence of the cold transfer buffer and 100% methanol.

This electroblotting method uses an electric current to migrate the negatively-charged proteins from the gel towards the positively-charged electrode into PVDF or nitrocellulose membrane, where they bind on basis of hydrophobic and charge interactions.

In order to check the effectiveness of the transfer, the membrane was stained by Ponceau Red solution for 5 minutes and rinsed with ddH_2O until the bands became well-defined. After registering, the membrane was distained completely by repeated washing with TBST.

5.3.5 Blocking

The membrane was incubated in the blocking solution consisting of 5% non-fat dry milk (NFDM) in TBST for 1 hour at room temperature under agitation in order to reduce unspecific binding of the antibodies.

5.3.6 Incubation with primary antibody

Detection with antibodies is a sensitive method based on the normal immune reaction components, when antibodies bind to the specific antigen. Membranes were incubated with monoclonal rabbit antibodies detecting ERK 1/2 and phosphorylated ERK 1/2 (p-ERK 1/2) both at a dilution of 1:500 in 5% BSA in TBST after previously tested most appropriate dilution. The membrane was incubated with antibody solution overnight at 4°C under gentle agitation. In between the two detections, the membranes were stripped in mild stripping buffer.

5.3.7 Incubation with secondary antibody

After washing of the membranes 3 times 10 minutes with TBST at room temperature to remove unbound primary antibody, the membranes were incubated with a specific horseradish peroxidase-conjugated (HRP-conjugated) anti-rabbit IgG secondary antibody at a dilution of 1:1000 in 5% NFDM in TBST for 1 hour at room temperature and washed again 3 times 10 minutes with TBST at room temperature.

Secondary antibody is directed against the primary antibody and is usually linked to an enzyme, such as horseradish peroxidase catalyzing reaction, which products provide detectable luminescence.

5.3.8 Development

Enhanced Chemiluminescence (ECL) detection system was used to visualize the bands of proteins, where luminol is used as a substrate, which is converted by horseradish peroxidase to a light releasing substance. Following manufacturer's instructions for HRP substrate preparation, a sensitive sheet of photographic film was placed on the membrane surface and film was developed using automated x-ray film processor to detect and fix the signal. Dark bands corresponding to the detected protein of interest appear on the developed film, which density is in proportion of the amount of protein. Densitometric analysis of bands was carried out using ImageJ software. Absolute band intensity values of phosphorylated protein bands (p-ERK 1/2) were then divided by those for total protein (ERK 1/2) to generate a ratio. Band densities in different lanes were then compared providing information on relative abundance of the protein of interest.

5.3.9 Membrane stripping

In order to detect the protein with another antibody, the combination of detergent and heat was used to disrupt the antigen-antibody interaction. Therefore, membranes were incubated in a mild stripping buffer for 30 minutes at 50°C in agitation. Three times washing with TBST was necessary before blocking and incubating with another antibody.

5.4 Vascular permeability assay

Plasma extravasation is one of the characteristic symptoms associated with inflammation. It is a process in which proteins and cells leak out of postcapillary venules accompanied by fluid. Evans blue dye has a very high affinity for serum albumin, which is often used to quantitatively assess the extent of extravasation as a symptom of inflammation.

The animals were anesthetized with a sleeping mix injected intraperitoneally depending on the body weight of the mouse (3 µl/g). The 1% Evans blue in PBS was subsequently administered intraorbitally in the total volume of 50 µl for each mouse. 5

minutes after the animal turned blue, 20 µl of 3 µM LPI was injected intraplantary into one hind paw, whereas contralateral paw was used as a control. PBS injection was used as a negative and 1 µg of histamine injection as a positive control in the test results evaluation. After 10 minutes of different treatments, the mice were killed by cervical dislocation and tissue samples containing a 12 mm^2 paw skin were incubated in 200 µl of formamide at 55°C for 48 hours. Afterwards, the amount of Evans blue was determined spectrophotometrically at the maximum absorption of 595 nm and expressed as a number of nanograms per 1 mm^2 of the skin.

5.5 Staining

5.5.1 Hematoxylin and eosin staining

5.5.1.1 Treatment of animals and preparation for staining

After the 30 seconds isofluran anesthesia, the animals were intraplantary injected with 3 µM, 300 µM LPI, CFA and PBS into one hind paw. The animals were anesthetized with CO_2 after 10 minutes, fixed on the cork plate and after median thoracotomy the heart was perfused with 20 ml of PBS and 20 ml of 4% PFA subsequently in order to fix the tissue proteins. The skin of the total area of 12 mm^2 was then removed from each paw and put in 4% PFA solution, whereas the contralateral paws were used as a control.

5.5.1.2 Cutting

The paw skin was stored in 30% sucrose overnight before the cutting was performed. Cryomicrotom set at -25°C and thickness of 25 µm was used to cut the skin tissue. The paw skin was fixed in the correct orientation with the help of tissue freezing medium. The sections were collected on previously coated polysine (0.1 %) slides and stored at -20°C.

5.5.1.3 Staining

Hematoxylin and eosin staining (H and E staining) is a widely used staining method in histology. This method enables differentiation of the structures of tissue and determines the changes in normal or inflamed or other pathologicaly changed

structures. The actual staining consists of two main parts - nuclear staining provided by hematoxylin, which stains the nuclei in dark blue and cytoplasm staining, which is performed by xanthenes dye and it stains the cytoplasm in red.

Slides with the cryosections were dried for 30 minutes at room temperature. As non-paraffin sections were used, the deparaffination and rehydration was not necessary. We proceeded with 1% acidic acid in ddH_2O to incubate the slides for 20 seconds. Then hematoxylin solution was used for 4 minutes, which was subsequently washed out from slides with warm tapped water for 2 minutes. The intensity of staining was controlled under microscope and the time was adjusted for optimal visibility. After hematoxylin staining, the eosin solution was poured for 5 seconds on each slide and 1% acidic acid in ddH_2O afterwards. The sections were dehydrated with 70%, 80%, 95% and 99% ethanol series for 2 minutes and with xylol at the end 2 times for 5 minutes. The dried slides were embedded with coverslips using Mowiol ensuring no bubbles. Images were obtained using Leica microscope.

5.5.2 DAB staining

The slides with cryosections were dried for 30 minutes at room temperature. 1% H_2O_2 in PBS/Methanol was used for 15 minutes at room temperature in order to block endogenous peroxidise activity. After washing the slides for 10 minutes with 0.2% PBST, the unspecific binding was blocked with 10% normal horse serum in PBS (NHS/PBS) for 40 minutes at room temperature. The sections were then incubated with biotinylated anti Gr-1 antibody in a dilution of 1:500 in blocking solution overnight at room temperature protected from light. Rat IgG bitotinylated antibody in a dilution of 1:200 in blocking solution was used to incubate the negative control slides.

The following day the slides were rinsed 2 times for 10 minutes with 10% NHS and 10 minutes in PBS and afterwards they were incubated with the ABC complex prepared following manufacturer's instructions by mixing 200 µl of PBS, 4 µl of reagent A and 4 µl of reagent B for each slide. After 30 minutes of incubation, the slides were washed 3 times for 10 minutes with PBS. 500 µl of ddH_2O, 10 µl of buffer stock solution, 20 µl of DAB and 10 µl of H_2O_2 were used to prepare the DAB solution. Afterwards, this solution was applied on the slides whereas the intensity of staining was controlled

under the microscope until the sections became dark. After achieving sufficient staining, the reaction was stopped by applying ddH_2O on each slide. The dried slides were embedded with coverslips using Mowiol. Images were obtained using Leica microscope.

5.5.3 Toluidine blue staining

Toluidine blue staining technique is a method used to visualize myelin-associated glycoprotein in order to investigate the peripheral nerve morphology or pathological changes associated with demyelination. Toluidine blue solution stains myelin sheaths bluish purple and neuronal nuclei light blue with dark granules of chromatin.

5.5.3.1 Treatment of animals and preparation for staining

The animals were anesthetized with a sleeping mix injected intraperitoneally depending on the body weight of mouse (3 µl/g). The sciatic nerve of the right hind limb was surgically exposed at the level of the high thigh through a small incision and 1 µl of 3 µM, 300 µM LPI or PBS was subsequently injected with into the sciatic nerve. The incision was sutured and mice were allowed to recover. After 24 hours, the animals were asphyxiated with CO_2, fixed on the cork plate and after median thoracotomy, the heart was perfused with 20 ml of PBS and 20 ml of 4% PFA subsequently in order to fix the tissue proteins. Both the right and left sciatic nerves were then removed and stored in 4% PFA.

5.5.3.2 Cutting and staining

2 µm thin sections were cut and stained with alkaline toluidine blue. At first, the sections were deparaffinized at 65°C in an oven, followed by 15 minute treatment with xylene. Subsequently, the sections were hydrated using xylene for 5 minutes twice and in 100%, 90%, 70% and 50% alcohol series for 2 minutes in each dilution. The sections were stained for 5 minutes with previously prepared toluidine blue staining solution consisting of 1g toluidine blue in 100 ml of 1% sodium borate. They were then washed in water and dehydrated with 70%, 90% and 100% alcohol series for 30 seconds, treated with xylene for 30 seconds, air-dried and mounted with mowiol. The stained sections were observed and images were obtained using Leica light microscope.

5.6 Statistical analysis

All data are presented as mean ± SEM. Statistical comparison of two groups was provided using two-tailed student t-test whereas multiple groups comparison by using one way analysis of variance (ANOVA) followed by Neuman-Keuls test. $P<0.05$ was considered to be statistically significant difference.

6 RESULTS

6.1 LPI induced ERK activation in DRG culture

We first examined the effect of LPI on ERK phosphorylation in the DRG culture, since ERK is a major downstream target of various intracellular signaling pathways. Also, LPI has been suggested to activate ERK 1/2 signaling in GPR55 expressing HEK293 cells (Oka et al. 2007). Although GPR55 is expressed in sensory neurons of the dorsal root ganglion (Lauckner et al. 2008), it is not very clear if GPR55 ligand LPI can activate its downstream signaling cascades also in the DRG neurons. Thus, we examined whether the expression of ERK 1/2 and of its active form phospho-ERK would be altered after LPI treatment in DRG neurons.

Western blot experiments were performed in order to detect changes in the expression of ERK and p-ERK proteins in neuron-enriched DRG culture 15 minutes after 3 µM LPI treatment. After separation of the proteins using the SDS-PAGE electrophoresis, proteins were blotted onto a nitrocellulose membrane and p-ERK 1/2 was detected using phospho-specific antibody. Subsequently, blots were stripped and re-probed with the antibody detecting both phosphorylated and non-phosphorylated forms of ERK 1/2. The p-ERK signal intensity of the DRG culture treated with 3 µM LPI was compared with signal intensity of the DRG culture treated with the vehicle.

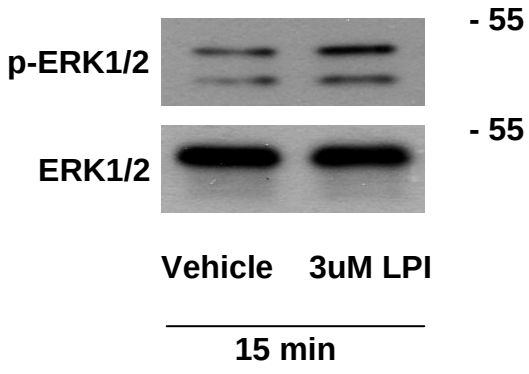


Figure 6.1.1. Western blot of protein lysates from neuron-enriched DRG culture showing ERK phosphorylation 15 minutes after application of LPI.

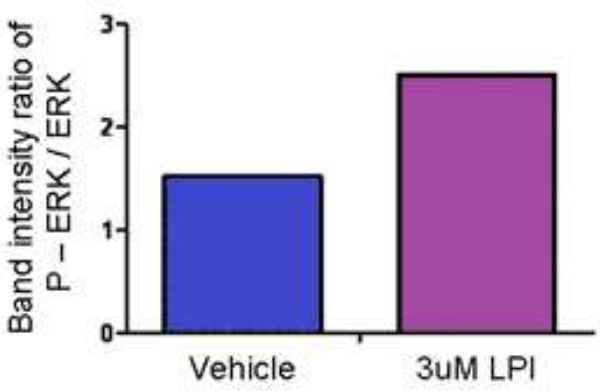


Figure 6.1.2. Quantification of ERK phosphorylation in neuron enriched DRG culture 15 minutes after application of LPI. The histograms quantify the changes of anti-p-ERK 1/2 antibody signals of vehicle and 3 µM LPI-treated DRG culture, showing an increase of phosphorylation of ERK 1/2 15 minutes after 3 µM LPI treatment. Changes were measured with optical density values expressed in A.U.

The western blot analysis revealed that stimulation of neuron-enriched DRG culture with 3 µM LPI increases the phosphorylation of ERK 1/2 15 minutes after treatment compared with vehicle treated DRG cultures under equal protein loading conditions assessed with total ERK 1/2 as demonstrated by western blots (Figure 6.1.1.) and its quantification (Figure 6.1.2).

This experiment indicates that LPI can directly activate neurons of DRGs. This observation also led us to an important question; whether LPI released from the cancer tissue could cause the activation of nociceptive terminals in the cancer environment and therefore lead to cancer induced pain.

6.2 LPI induced Hyperalgesia

In order to test our hypothesis that the bioactive lysophospholipid LPI released from the tumor environment can possibly evoke mechanical hypersensitivity, we injected LPI into the plantar surface of the hind paw of the mice. A single intraplantar injection of LPI led to long lasting mechanical hyperalgesia in a dose dependent manner without eliciting any swelling or reddening throughout the experiment (Figure 6.1). On the other hand, injection of vehicle alone did not lead to any significant change in the response frequency.

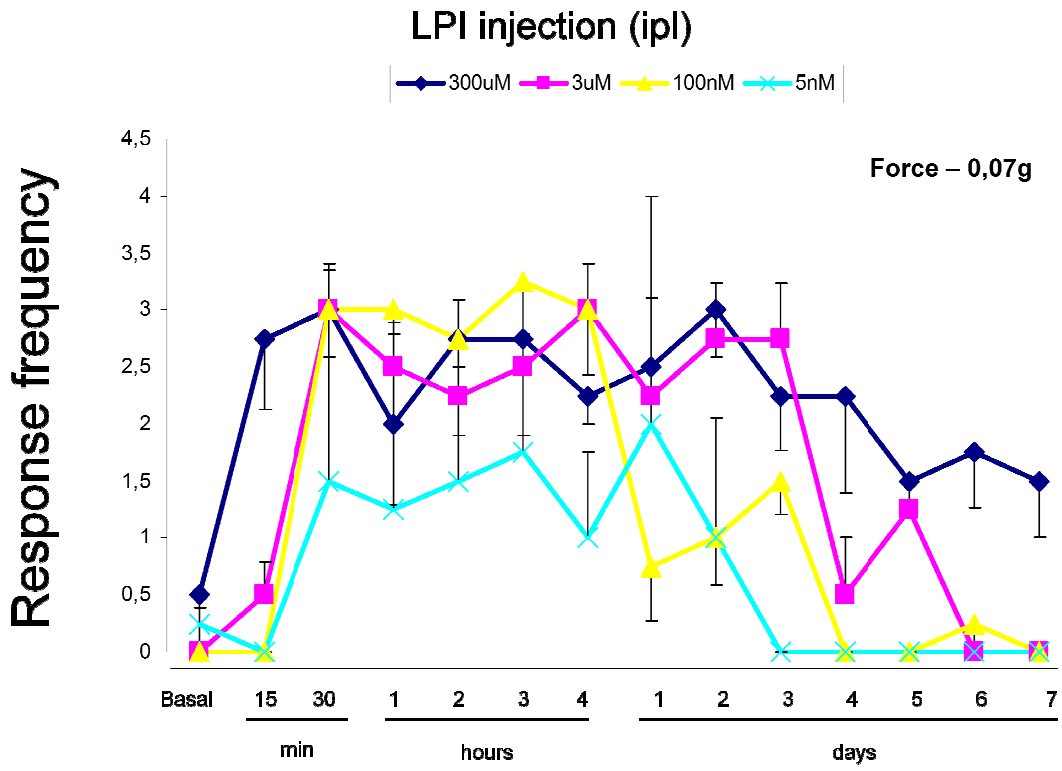


Figure 6.2. LPI induced mechanical hyperalgesia

An upward and leftward shift in the above stimulus-response curve indicates the hypersensitivity to mechanical force applied using von Frey filament to the hind paw of the animal.

6.3 Hematoxylin and eosin staining

Although there were no obvious inflammatory responses like reddening or oedema present after LPI injection, we performed H and E staining to visualize possible inflammatory changes (leukocyte infiltration, dilated blood vessels, etc.) in the skin of the plantar surface of the hind paw after LPI treatment. However, we did not observe any inflammatory changes in the skin after the injection of the same doses of LPI, which were previously used to evoke hyperalgesia. On the other hand, there was a large amount of infiltrated leukocytes and dilated blood vessels observed 24 hours after injection of CFA, which was used as a positive control (Figure 6.3.).

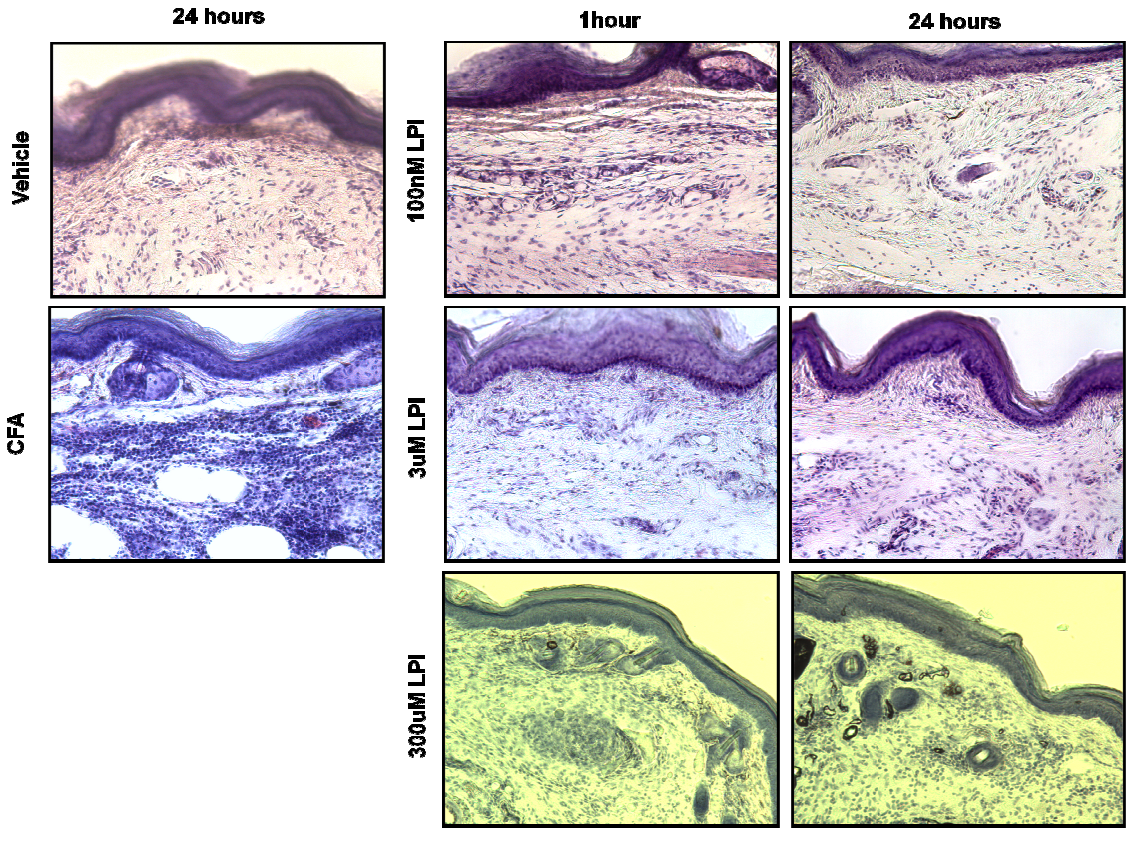


Figure 6.3. Representative sections of H and E stained paw skin. Magnification 10×

6.4 Neutrophil invasion

To further characterize the accurate inflammatory state after LPI treatment, we labeled neutrophils of the paw skin sections using Gr-1 antibody, a marker for the same and we stained them using DAB system. We found very few infiltrated neutrophils after the application of 300 µm LPI and hardly any neutrophils in other groups (3 µM LPI, 100 nM LPI and vehicle). In contrast, large amount of neutrophils was found in the skin 24 hours after CFA injection (Figure 6.4.1).

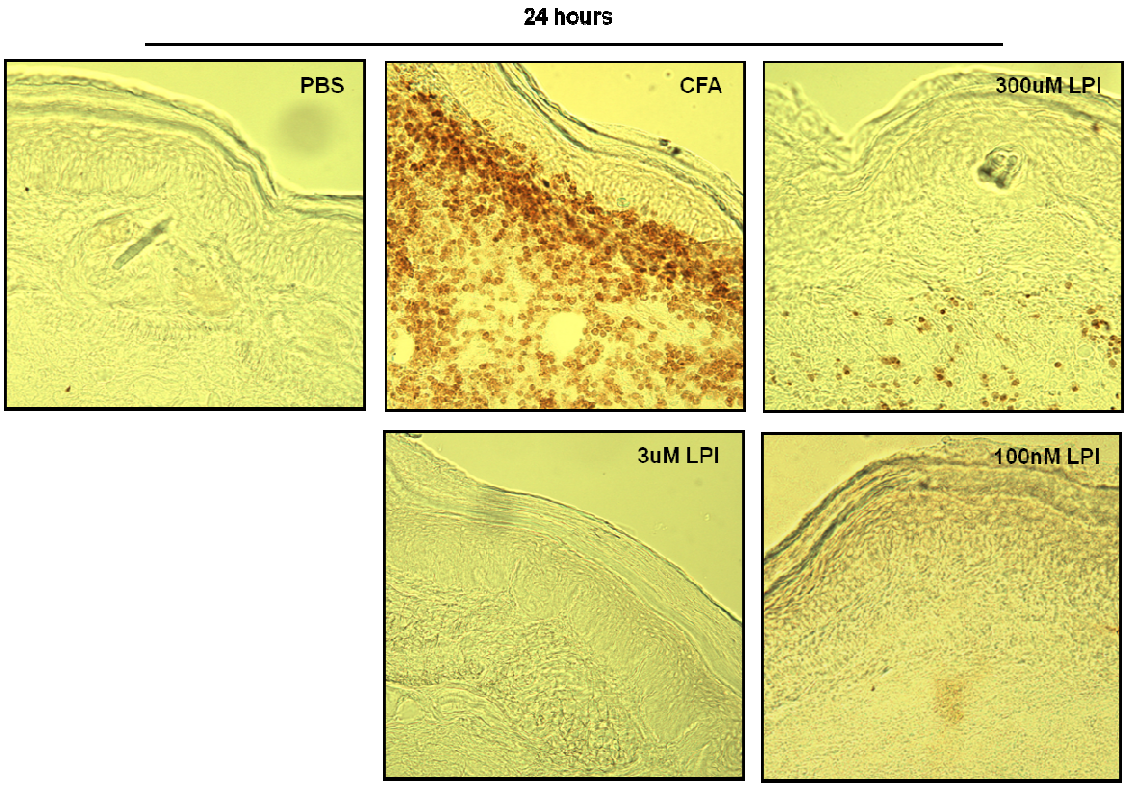


Figure 6.4.1. Representative sections of DAB stained paw skin. Magnification 20×

Stained invaded cells were subsequently counted manually and quantified using Graph prim 5 software in order to accurately determine the number of neutrophils, which have traversed into the injected area (Figure 6.4.2.).

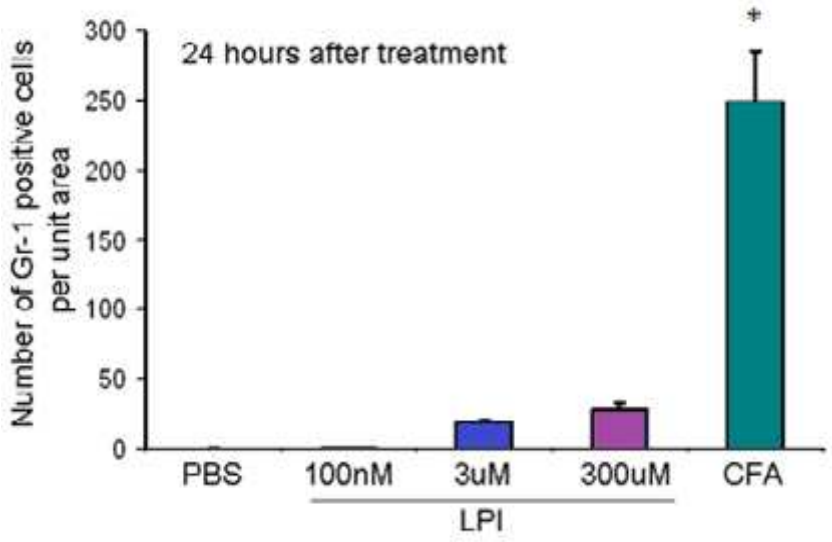


Figure 6.4.2. Quantitative analysis of neutrophil infiltration. * P < 0.001

The above H and E and Gr-1 staining clearly suggest that LPI does not mediate mechanical hypersensitivity by inducing inflammation.

6.5 Extravasation

The vascular events associated with inflammation could involve changes in blood flow and alterations in permeability resulting in oedema and possibly also lead to the mechanical hypersensitivity. Thus, we determined the extend of extravasation after intraplantar injection of PBS, 3 µM LPI and 1 µg of histamine using Evans blue dye test.

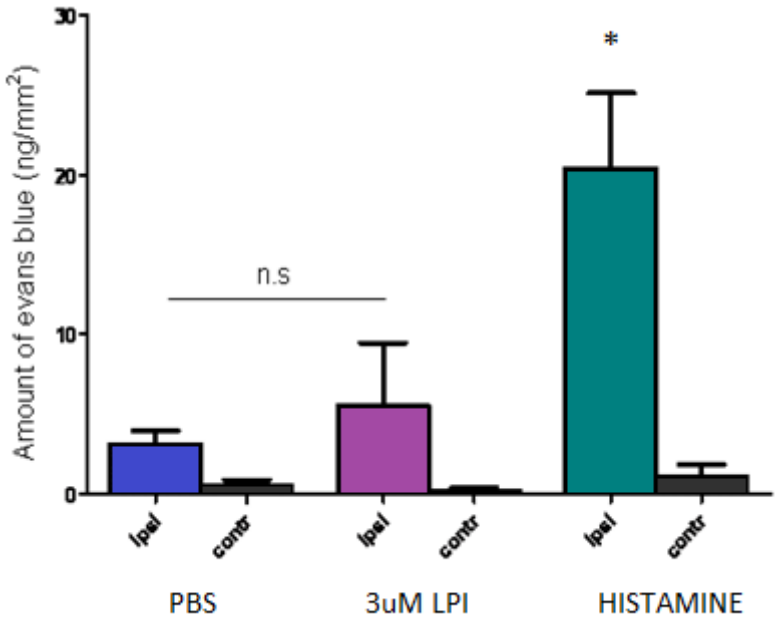


Figure 6.5. Vascular permeability changes associated with intraplantar injection of PBS, 3 µM LPI and histamine. *P< 0.001, n.s P = 0.5631

We observed that neither treating mice with 3 µM LPI nor with the vehicle led to accumulation of systemically injected Evans blue dye in the area of the skin. In contrast, injection of 1 µg of histamine led to a strong plasma extravasation. This experiment also indicates that LPI does not bring about the mechanical hypersensitivity by plasma extravasation (neurogenic inflammation).

6.6 Demyelination

It has been reported, that neuropathic pain is associated with aberrant demyelination (Gillespie et al. 2000). In order to investigate the mechanism, through which LPI possibly mediates its pronociceptive activity, we examined the myelination status of mice sciatic nerves after intranerval LPI injection using Toluidine blue staining and light microscopic analysis (Figure 6.6.).

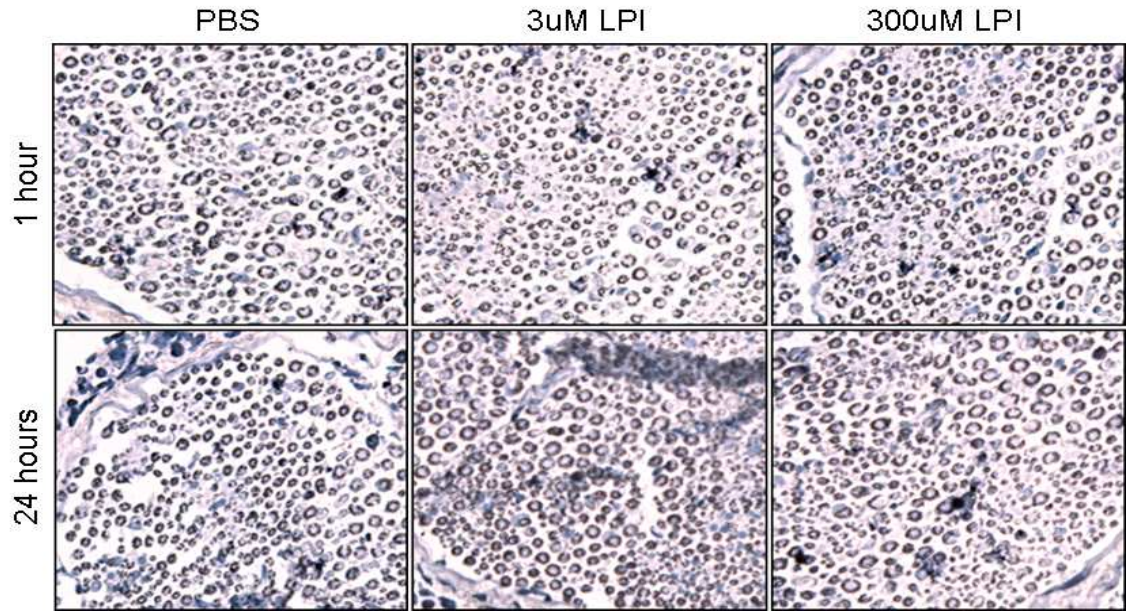


Figure 6.6. Representative sections of TB stained sciatic nerves. Magnification 20×

According to our observation, treatment with LPI was not followed by demyelination of the injected sciatic nerves. This suggests that LPI does not elicit mechanical hypersensitivity by affecting the myelination of sensory neurons.

7 DISCUSSION

GPR55 has been recently identified as a novel cannabinoid receptor with atypical responsiveness to cannabinoid ligands (Ryberg et al. 2007; Kapur et al. 2009). Recent study with GPR55 expressing HEK293 cells has also shown that GPR55 is activated by the endogenous lipid signaling molecule LPI (Oka et al. 2007) and that it is expressed among other tissues also on sensory neurons (Lauckner et al. 2008). In addition, LPI is known to be produced by variety of cancer tissues (Falasca et al. 1998), suggesting the hypothesis that LPI might be one of the factors corresponding to the development of neuropathic cancer pain.

One of the pathways involved in mitogenic signaling induced by various GPCRs is the pathway of extracellular-regulated protein kinase (ERK), which belongs to the mitogen-activated protein kinase (MAPK) signaling cascades (Cargnello and Roux). Stimulation of GPR55 by LPI is known to induce rapid phosphorylation of ERK 1/2, which was revealed at the study using HEK293 transfected cells (Henstridge et al.; Oka et al. 2007). In agreement with this study, using WB method we investigated that LPI treatment leads to activation of ERK signaling pathway in DRG cell culture (Figure 6.1.1.). This suggests that LPI can directly activate sensory neurons *in vitro*, which is possibly one of the pathways through which LPI exerts its properties on sensory neurons *in vivo*.

In order to establish the physiological or pathophysiological function of LPI, further investigation needed to be performed, especially due to very limited information providing the ability of LPI to induce pain *in vivo*. Research performed by Staton et al. (2008) revealed that GPR55 signaling pathway plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain, where they demonstrated that GPR55 deficient mice failed to develop inflammatory mechanical hyperalgesia following intraplantar administration of CFA up to 14 days post-injection. Following this study we investigated that intraplantar administration of LPI to the wild-type mice leads to long lasting mechanical hyperalgesia in a dose dependent manner without

eliciting any signs of inflammation (Figure 6.2.), which clearly supports previously assumed pronociceptive properties of LPI.

The cardinal signs of inflammation involve heat (calor), redness (rubor), swelling (tumor) and pain (dolor), often accompanied by loss of function (function laesa) (Kumar et al. 2010). Even though we observed no signs of inflammation after intraplantar LPI injection in previous experiment, the study of Staton et al. (2008) showed the involvement of GPR55 signaling pathway in inflammatory pain and thus we performed H and E staining of injected paw skin in order to visualize possible inflammatory changes on the cellular level following LPI injection. Our observation led us to a conclusion that intraplantar LPI administration does not elicit any visual pathological changes in the skin structure with any massive cell invasion nor vasodilatation (Figure 6.3.).

The major cell types producing substances that contribute to the acute inflammatory reaction are platelets, neutrophils, monocytes/macrophages and mast cells, while neutrophils constitute the predominant cell type in the early inflammatory reaction (Kumar et al. 2010). Thus, we used Gr-1 antibody to specifically label neutrophils and DAB staining of the histological sections of paw skin to visualize the cellular inflammatory changes following intraplantar LPI administration (Figure 6.4.1.). Quantitative analysis of the present number of cells revealed non-significant increase of neutrophils in the injected area (Figure 6.4.2.), which also supports our previous experiment observation.

As previously mentioned, acute inflammatory reaction is associated with massive vasodilatation leading to an increase in blood flow reflected by the heat and redness of the tissue. The vasodilatation is followed by alterations in microvascular permeability resulting in leakage of plasma from the vasculature (Kumar et al. 2010). In order to assess the microvascular permeability changes after LPI administration we performed vascular permeability assay which revealed no aberrant extravasation after LPI administration (Figure 6.5.).

All these observations concerning the experiments investigating the mechanism of action of LPI indicate that LPI does not mediate its pronociceptive effect by inducing inflammation.

It has been reported that demyelination and subsequent physical contact of nerve fibers might be one of the mechanisms responsible for neuropathic pain (Osterberg et al. 2005). In addition, lysophosphatidic acid (LPA), a closely related lysophospholipid of LPI can cause demyelination and neuropathic pain in mice and blocking of the LPA signaling through its receptor was shown to reverse the neuropathic pain (Inoue et al. 2004). It has also been described that the enzyme autotaxin converting various lysophospholipids into LPA is present in the plasma (Inoue et al. 2008). These studies raise the possibility that injected LPI might be *in vivo* converted into LPA, leading to demyelination and mechanical hypersensitivity. Thus, we investigated the patho-physiological changes in myelinated axons of sciatic nerve induced by LPI injection using Toluidine blue staining method. Observation of visualized myelinated fibers clearly indicated that no aberrant demyelination is associated with LPI injection (Figure 6.6.), which does not support previous hypothesis about LPI converting into LPA *in vivo.* However, blocking of LPA receptor downstream and subsequent LPI stimulation can also help to reveal whether LPI does not exert its pronociceptive effect through the conversion to LPA.

8 CONCLUSION

Although LPI and its receptor GPR55 is expressed in the nervous system, very little is known about their function *in vivo*. We found that LPI can directly activate nociceptors by activating ERK signaling pathway *in vitro* and that LPI injected in the periphery leads to a mechanical hypersensitivity in mice in a dose dependent manner. In addition, the results of this study demonstrate that LPI does not elicit any inflammation, demyelination or extravasation.

Selective blocking of the LPI-GPR55 pathway may represent a novel perspective in treatment and prevention not only of cancer and non-cancer pain but it may also contribute to reducing tumor growth and metastasis. Therefore, the elucidation of physiological functions of GPR55 and its endogenous ligand LPI is likely to provide better treatment options in future.

9 ABBREVIATIONS

A.U.	Arbitrary unit
Ab	Antibody
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APS	Ammonium persulfate
AraC	Cytarabine
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid receptor
CBD	Cannabidiol
CBN	Cannabinol
CFA	Complete Freund's adjuvant
CNS	Central nervous system
DAB	Diaminobenzidine
ddH_2O	Double distilled water
DMEM	Dulbecco´s modified eagle medium
DNAse	Deoxyribonuclease
DPBS	Dulbecco´s phosphate buffered saline
DRG	Dorsal root ganglia
DTT	Dithiotreitol
ECL	Enhanced Chemiluminescence
ERK	Extracellular signal regulated kinase
FBS	Fetal bovine serum
GPCR	G-protein-coupled receptor
GPR55	G-protein-coupled receptor 55
H and E	Hematoxylin and eosin
HEK cells	Human embryonic kidney cells

HRP	Horseradish peroxidase
IASP	International Association for the Study of Pain
IgG	Immunoglobulin G
LPA	Lysophosphatidyl acid
LPI	Lysophosphatidylinositol
MAPK	Mitogen activated protein kinase
mGluR	Metabotropic glutamate receptor
NFAT	Nuclear factor of activated T cells
NFDM	Non-fat dry milk
NGF	Nerve growth factor
NHS	Normal horse serum
NMDA	N-methyl-D-aspartate
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20
P/S	Penicillin/Streptomycin
PFA	Paraformaldehyde
PVDF	Polyvinylidene difluoride
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIP	Stock isotonic Percoll
TB	Toluidine blue
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TEMED	Tetramethylethylenediamine
THC	Δ^9-tetrahydrocannabinol
Tris	Tris-(hydroxymethyl)-aminomethan
WB	Western blot

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